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Transcriptional response of blood leukocytes from turkeys challenged with Salmonella enterica serovar Typhimurium UK1

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Abstract

Non-typhoidal *Salmonella* is one of the most common causes of bacterial foodborne disease and consumption of contaminated poultry products, including turkey, is one source of exposure. Minimizing *Salmonella* colonization of commercial turkeys could decrease the incidence of *Salmonella*-associated human foodborne illness. Understanding host responses to these bacteria is critical in developing strategies to minimize colonization and reduce food safety risk. In this study, we evaluated bacterial load and blood leukocyte transcriptomic responses of 3-week-old turkeys challenged with the *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) UK1 strain. Turkeys (n = 8/dose) were inoculated by oral gavage with 108 or 1010 colony forming units (CFU) of *S.* Typhimurium UK1, and fecal shedding and tissue colonization were measured across multiple days post-inoculation (dpi). Fecal shedding was 1–2 log10 higher in the 1010 CFU group than the 108 CFU group, but both doses effectively colonized the crop, spleen, ileum, cecum, colon, bursa of Fabricius and cloaca without causing any detectable clinical signs in either group of birds. Blood leukocytes were isolated from a subset of the birds (n = 3–4/dpi) both pre-inoculation (0 dpi) and 2 dpi with 1010 CFU and their transcriptomic responses assayed by RNA-sequencing (RNA-seq). At 2 dpi, 647 genes had significant differential expression (DE), including large increases in expression of immune genes such as *CCAH221*, *IL4I1*, *LYZ*, *IL13RA2*, *IL22RA2*, and *ACOD1*. IL1β was predicted as a major regulator of DE in the leukocytes, which was predicted to activate cell migration, phagocytosis and proliferation, and to impact the STAT3 and toll-like receptor pathways. These analyses revealed genes and pathways by which turkey blood leukocytes responded to the pathogen and can provide potential targets for developing intervention strategies or diagnostic assays to mitigate *S.* Typhimurium colonization in turkeys.

Keywords

Salmonella enterica serovar Typhimurium, Foodborne pathogen, Turkey, Colonization, RNA-seq, Leukocyte

Disciplines

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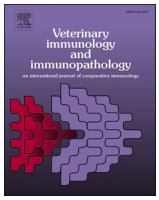

Transcriptional response of blood leukocytes from turkeys challenged with *Salmonella enterica* serovar Typhimurium UK1

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ABSTRACT

Non-typhoidal *Salmonella* is one of the most common causes of bacterial foodborne disease and consumption of contaminated poultry products, including turkey, is one source of exposure. Minimizing *Salmonella* colonization of commercial turkeys could decrease the incidence of *Salmonella*-associated human foodborne illness. Understanding host responses to these bacteria is critical in developing strategies to minimize colonization and reduce food safety risk. In this study, we evaluated bacterial load and blood leukocyte transcriptomic responses of 3-week-old turkeys challenged with the *Salmonella enterica* serovar Typhimurium (*S*. Typhimurium) UK1 strain. Turkeys (n = 8/dose) were inoculated by oral gavage with 10^8 or 10^{10} colony forming units (CFU) of *S*. Typhimurium UK1, and fecal shedding and tissue colonization were measured across multiple days post-inoculation (dpi). Fecal shedding was 1–2 $\log_{10}$ higher in the 10^{10} CFU group than the 10^8 CFU group, but both doses effectively colonized the crop, spleen, ileum, cecum, colon, bursa of Fabricius and cloaca without causing any detectable clinical signs in either group of birds. Blood leukocytes were isolated from a subset of the birds (n = 3–4/dpi) both pre-inoculation (0 dpi) and 2 dpi with 10^{10} CFU and their transcriptomic responses assayed by RNA-sequencing (RNA-seq). At 2 dpi, 647 genes had significant differential expression (DE), including large increases in expression of immune genes such as *CCAH221*, *IL4I1*, *LYZ*, *IL13RA2*, *IL22RA2*, and *ACOD1*. IL1β was predicted as a major regulator of DE in the leukocytes, which was predicted to activate cell migration, phagocytosis and proliferation, and to impact the STAT3 and toll-like receptor pathways. These analyses revealed genes and pathways by which turkey blood leukocytes responded to the pathogen and can provide potential targets for developing intervention strategies or diagnostic assays to mitigate *S*. Typhimurium colonization in turkeys.

1. Introduction

Non-typhoidal *Salmonella* is a leading cause of bacterial foodborne disease, resulting in an estimated 1 million illnesses in the U.S. annually (Scallan et al., 2011). Food animals are often asymptomatically colonized with *Salmonella*, resulting in unintentional contamination of the food supply (Stevens et al., 2009). Poultry is a common source of *Salmonella*-associated foodborne illness, and approximately six percent of illnesses are attributed to consumption of improperly prepared or handled turkey products (IFSAC, 2019). Due to its impact on food safety, *Salmonella* is listed on the top ten current health and industry issues facing the turkey industry (Forebel and Clark, 2019).

Salmonella enterica serovar Typhimurium (*S*. Typhimurium) was found by the Centers for Disease Control and Prevention to be among the top 3 most frequent non-typhoidal *Salmonella* serovars to cause human foodborne illness in the U.S. (CDC, 2017), and was the most common serovar resulting in mortality (Jones et al., 2008; Kennedy et al., 2004). Limiting colonization of food animals by *Salmonella* serovars such as *S*. Typhimurium could reduce human foodborne disease, and investigating host-pathogen interactions may reveal potential strategies of colonization intervention. Transcriptome-level responses within *Salmonella*--colonized chicken tissues such as the spleen (Li et al., 2018; Ma et al.,

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2014; Matulova et al., 2012; Zhou and Lamont, 2007), liver (Coble et al., 2013), cecum (Higgins et al., 2011; Ma et al., 2014; Matulova et al., 2013), and cecal tonsil (Wang et al., 2019) have been evaluated by RNA-sequencing (RNA-seq) or microarrays. However, the majority of these studies focused on other *Salmonella* serovars and, to our knowledge, only our group has used transcriptomics to investigate *Salmonella*-challenged turkeys and, specifically, their peripheral blood leukocytes (Bearson et al., 2017, 2019). As blood can be easily and repeatably collected from an animal, circulating leukocytes are an ideal sample for developing biomarkers to predict colonization, or for assaying responses to improve vaccines.

In the current study, we evaluated the colonization potential of the *S.* Typhimurium UK1 strain and blood leukocyte transcriptional response of turkeys to the pathogen. Host responses to *S.* Typhimurium UK1 are especially relevant due to 1) the virulent nature of the UK1 strain, which has been well-characterized in multiple animal species and 2) the attenuated live vaccines that have been derived from UK1 for multiple animal species (Curtiss et al., 1991; Luo et al., 2012; Zhang et al., 1999). In our analysis, *S.* Typhimurium UK1 efficiently colonized 3-week old turkeys and prompted a robust host transcriptional response without producing clinical disease.

2. Material and methods

2.1. Ethics statement

This animal experiment was approved and conducted according to the regulations established by the National Animal Disease Center Institutional Animal Care and Use Committee.

2.2. Bacterial strains and selective medium

A nalidixic acid resistant *S.* Typhimurium UK1 strain (SB 377) (Bearson et al., 2016) was used in this study. The inoculum was prepared by growing the strain from a single colony statically in LB broth (Invitrogen by Life Technologies, Carlsbad, CA, USA) containing 30 μg/ml nalidixic acid (Sigma-Aldrich, Inc., St. Louis, MO, USA) at 37 °C for approximately 24 h. The culture was pelleted and resuspended in sterile phosphate buffered saline (PBS) (Sigma Life Sciences, St. Louis, MO, USA) at 1/10th of the original volume to achieve 1×10^{10} colony forming units (CFU); a portion of the 10^{10} CFU inoculum was diluted 1:100 in PBS to achieve the 1×10^{8} CFU inoculum. Bacterial growth medium for culture of *S.* Typhimurium UK1 from turkey samples was XLT-4 (Becton, Dickinson & Company, Sparks, MD, USA) containing 30 μg/ml nalidixic acid.

2.3. Animal trial and sample processing

One-day old Hybrid jake (male) turkey poults ($n = 16$) were obtained from a commercial farm, and co-housed in a single ABSL-2 room. Throughout the study, poults were fed a turkey poult starter ration and had water available *ad libitum*. Qualitative bacteriology (Bearson et al., 2010) did not detect *Salmonella* in the fecal samples of the group pen. At two weeks of age, turkeys were transferred into individual isolated cages within an ABSL-2 treatment room, and at 3 weeks of age, inoculated by oral gavage with 1 mL of 1×10^{8} ($n = 8$) or 1×10^{10} ($n = 8$) CFU of *S.* Typhimurium UK1 strain (SB 377). Using a Medline thermometer model # MDS9850B (Mundelein, IL, USA), cloacal temperatures were measured at 0, 1, 2, and 3 days post-inoculation (dpi). Fecal samples were collected from individual turkeys (within 1 h of defecation on a clean pad on the cage floor) at 0, 1, 2, 3, 7, 10, and 14 dpi for *S.* Typhimurium UK1 enumeration using quantitative and qualitative bacteriology, as previously described (Bearson et al., 2016). All fecal samples post-inoculation were quantitatively positive (≥ 50 CFU/g). At 7 and 14 dpi, four turkeys randomly selected from each group (10^{8} CFU inoculated and 10^{10} CFU inoculated) were euthanized using barbiturates at label dose or carbon dioxide gas (based on weight). From each bird, 1 g of tissue samples from the crop, spleen, liver (7 dpi only), duodenum (14 dpi only), ileum, cecum, cloaca, colon (14 dpi only), and bursa of Fabricius (14 dpi only) were aseptically collected, including rinsing intestinal lumens with sterile PBS to remove the digesta, for *S.* Typhimurium UK1 detection and enumeration, as previously described (Bearson et al., 2016). Tissue samples that were negative by both quantitative and qualitative bacteriology were assigned 0 CFU/g, while those positive only by enrichment were assigned a random value between 1 and 20 CFU/g (limit of detection). Enumeration data from both fecal and tissue samples were $\log_{10}$ transformed. Using GraphPad Prism 5.01 (GraphPad Software, San Diego, CA, USA), statistical analysis comparing the two inoculation doses for fecal shedding and the level of tissue colonization of the crop, spleen, ileum, cecum, and cloaca was performed using a two-way analysis of variance with a Bonferroni posttest. For statistical analysis of fecal shedding over time within a given inoculation dose, a one-way analysis of variance with a Tukey's multiple comparison test was conducted. An unpaired *t*-test was used to determine statistical significance for tissue colonization levels of the crop, spleen, ileum, cecum, and cloaca comparing 7 and 14 dpi within an inoculation dose, and for the comparison between inoculation doses in the bursa and colon as these tissue samples were only obtained at a single time point. For all statistical tests listed above, significance was set at $P < 0.05$.

2.4. RNA isolation and sequencing from turkey blood leukocytes

Prior to inoculation (0 dpi) and 2 dpi with 10^{10} CFU *S.* Typhimurium UK1, blood was collected from the brachial vein of turkeys selected for RNA-seq (different birds at each dpi) into EDTA tubes and fractionated using the LeukoLOCK™ Fractionation & Stabilization Kit (Thermo Fisher Scientific, Waltham, MA, USA). As clinical signs were not evident in the turkeys given either 10^{8} or 10^{10} CFU, the 10^{10} dose was selected for global transcriptome analysis. Two dpi was selected as the post-inoculation timepoint because this is typically the acute stage of colonization and the highest level of *Salmonella* shedding. The LeukoLOCK™ Total RNA Isolation System (Thermo Fisher Scientific) was employed to extract total RNA from the leukocyte population in each sample, and an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA) was used to assess resulting RNA quality and quantity (average RNA integrity number = 7.2; range = 5.9–8.1). The TruSeq RNA Sample Prep Kit v2 (Illumina Inc., San Diego, CA, USA) was used to construct cDNA libraries for both 0 dpi ($n = 4$) and 2 dpi ($n = 4$) samples. The resulting uniquely indexed libraries were multiplexed into a single pool, seeded onto four lanes of a flow cell (*e.g.* four replicates/library), and sequenced using an Illumina HiSeq 2500 sequencer (Illumina Inc.) to produce 100-cycle paired-end reads at the Iowa State University DNA Facility (Ames, IA, USA). Sequencing of one of the 0 dpi samples was poor-quality and additional RNA was not available to repeat the sequencing; therefore, one less sample was analyzed for 0 dpi ($n = 3$) than 2 dpi ($n = 4$) in the downstream analyses.

2.5. Bioinformatic and differential expression (DE) analysis

CLC Genomic workbench 9.5.2 (QIAGEN, www.qiagenbioinformatics.com, Redwood City, CA, USA) was used to quality trim sequence reads and map the trimmed reads to the *Meleagris gallopavo* reference genome assembly 5.0 (GCA_000146605.3) (Dalloul et al., 2010). Gene expression levels (read counts) were calculated using reads that uniquely mapped to the turkey genome annotation (GCF_000146605.2; NCBI Annotation Release 102). Within CLC Genomics Workbench, edgeR methods (Robinson et al., 2010) were used for DE analysis. Read counts were normalized for library size by trimmed mean of M-values (TMM) method and used to perform an Exact Test for the negative binomial distribution to determine DE. The resulting fold changes were $\log_2$ transformed ($\log_2$FC) and *P*-values were False Discovery Rate (FDR) adjusted with the Benjamini-Hochburg method. Significance thresholds

were set at $|\log_2 FC| \geq 1.0$ and FDR < 0.01. A log-transformation of normalized read counts per million (cpm) from the 500 genes with the highest between-sample variance were also used for principal component analysis (PCA) in the R software package (R Core Team, 2019).

2.6. Pathway analysis

Canonical pathways, downstream functions, and upstream regulators associated with significant DE genes were identified using Ingenuity Pathway Analysis (IPA) (QIAGEN, www.qiagenbioinformatics.com). Because IPA does not recognize turkey gene IDs, $\log_2$FCs from significant DE genes were imported into IPA using gene symbol; gene symbols were obtained from the turkey genome annotation or from the closest chicken ortholog as identified by BLASTP 2.9.0+ (alignment score > 80) (Boratyn et al., 2013; Camacho et al., 2009). If chicken gene symbols were not recognized by IPA, the HomoloGene database was used to identify known human orthologs for the chicken genes, where present. Within IPA, significant associations to canonical pathways, downstream diseases and functions, and upstream regulators were determined using a Fisher's Exact test with multiple test correction (FDR < 0.05; Benjamini-Hochburg method) and, to incorporate directionality, examined for predicted activation (z-score ≥ 2.0), or inhibition (z-score $\leq$ -2.0) of the pathways/functions/regulators.

3. Results

3.1. Salmonella enterica serovar Typhimurium colonization and fecal shedding in turkeys

Three-week old male turkey poults were orally inoculated with 1×10^8 or 1×10^{10} CFU of *S.* Typhimurium UK1. Body (cloacal) temperature, fecal shedding of *Salmonella,* and tissue colonization were monitored. No significant difference in mean body temperature was detected when comparing the pre-inoculation body temperature (41.51 and 41.42 °C at 0 dpi) to a single post-inoculation time point (41.58 and 41.61 °C at 1 dpi, 41.39 and 41.69 °C at 2 dpi, and 41.42 and 41.57 °C at 3 dpi for the turkeys inoculated with 10^8 and 10^{10} CFU, respectively), indicating no significant increase in body temperature as a clinical sign of *S.* Typhimurium challenge in turkeys. Furthermore, no poults showed clinical signs of septicemia (depression, lethargy, anorexia), and no mortalities occurred as a result of the inoculation.

Fecal shedding of the UK1 strain ranged from 10^3-10^7 CFU/g of feces throughout the 14-day study (Fig. 1). Turkeys inoculated with 10^{10} CFU shed significantly more *Salmonella* than the 10^8 CFU inoculated turkeys at 1–3 dpi and 10 dpi (1–2 $\log_{10}$, $P < 0.05$); by 14 dpi, fecal shedding of the UK1 strain was similar between the two groups. From 1 dpi to 14 dpi, *Salmonella* shedding dropped 3 $\log_{10}$ in the 10^{10} CFU inoculated turkeys ($P < 0.05$) and 1 $\log_{10}$ in the 10^8 CFU inoculated turkeys ($P > 0.05$). Tissue colonization by *Salmonella* was assessed at 7 and 14 dpi (Fig. 2; **Supplemental Table S1**). *S.* Typhimurium was not detected in the liver at 7 dpi and, thus, the liver was not tested at 14 dpi; instead, the duodenum, colon and bursa of Fabricius were cultured at 14 dpi (**Supplemental Table S1**). The *S.* Typhimurium UK1 strain colonized the crop, spleen, ileum, cloaca, colon, and bursa of Fabricius at 1-1,000 CFU/g of tissue, and up to 10,000 CFU/g of cecal tissue (Fig. 2).

3.2. Transcriptome analysis of blood leukocytes from S. Typhimurium UK1 challenged turkeys

At 3 weeks of age, transcriptional analysis of turkey blood leukocytes was performed using RNA-seq on a subset of samples isolated pre-inoculation (0 dpi) and 2 dpi with 10^{10} CFU of *S.* Typhimurium UK1. Blood leukocytes represent circulating immune cells (heterophils, basophils, eosinophils, monocytes, and T- and B-lymphocytes) that can migrate to infected organs; expression profiling of these cells provides a global snapshot of the turkey's immune response to *Salmonella*

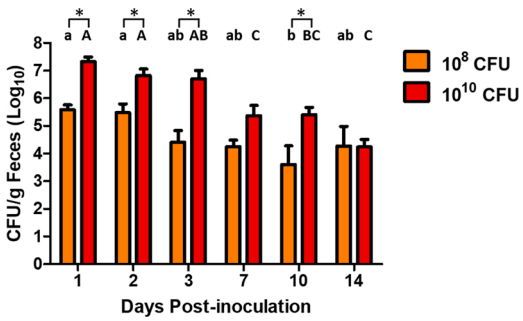

Fig. 1. Fecal shedding of *Salmonella enterica* serovar Typhimurium UK1 from turkeys.
Individually housed turkeys were inoculated with 10^8 ($n = 8$) or 10^{10} ($n = 8$) CFU of *S.* Typhimurium UK1 at 3 weeks of age. Feces were collected at the indicated days post-inoculation (dpi), and quantitative and qualitative bacteriology was performed to determine fecal shedding of *S.* Typhimurium. At 7 dpi, 4 poults were euthanized to culture for *Salmonella* colonization and dissemination; thus, fecal shedding data at 10 and 14 dpi were obtained from the 4 remaining poults. Error bars indicate SEM. Asterisks above the bars at a given time point denote significant differences in fecal shedding between the 10^8 and 10^{10} *Salmonella* doses ($P < 0.05$). Dissimilar letters above each bar indicate significant differences in fecal shedding over time within a treatment dose: 10^8 (a, b) and 10^{10} (A, B, C) ($P < 0.05$). 10^8 CFU (orange), 10^{10} CFU (red).

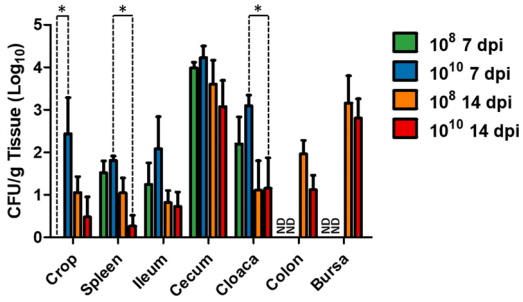

Fig. 2. Tissue colonization of *Salmonella enterica* serovar Typhimurium UK1 in turkeys.
Individually housed turkeys were inoculated with 10^8 ($n = 8$) or 10^{10} ($n = 8$) CFU of *S.* Typhimurium UK1 at 3 weeks of age. At 7 and 14 days post-inoculation (dpi), four turkeys from each group were euthanized. Quantitative and qualitative bacteriology was performed to determine *S.* Typhimurium colonization in the crop, spleen, ileum, cecum, colon, bursa of Fabricius, and cloaca. Error bars indicate SEM. Asterisks and dashed lines denote significant differences in *Salmonella* tissue colonization for the indicated tissues ($P < 0.05$). See **Supplemental Table S1** for the number of *Salmonella*-positive birds within each tissue for each day post-inoculation. ND = not done, 10^8 CFU 7 dpi (green), 10^{10} CFU 7 dpi (blue), 10^8 CFU 14 dpi (orange), 10^{10} CFU 14 dpi (red).

challenge. On average, 26.2 M reads per library (range from 13.7 M – 35.9 M) mapped uniquely to the turkey genome, and the reads cumulatively provide evidence for expression of 18,553 genes in turkey blood leukocytes. PCA on the 500 genes with the highest variability revealed that separation of the 0 dpi and 2 dpi libraries accounted for over 60 % of the variation in expression (Fig. 3).

Transcription of 647 genes was significantly altered ($|\log_2 FC| \geq 1.0$, FDR < 0.01) by *S.* Typhimurium inoculation (2 dpi compared to 0 dpi) (Fig. 4; **Supplemental Table S2**). Among the DE genes, 505 genes were up-regulated (including 22 unannotated gene predictions (identified by *LOC* number gene symbols) in turkey with no annotated chicken orthologs) and 142 down-regulated (including 5 *LOC* numbered genes without annotated chicken orthologs). Over half (54.3 %) of the significant DE genes had a small increase in expression after 2 days of challenge with *S.* Typhimurium UK1 ($1.0 \leq \log_2 FC \leq 2.0$) (Fig. 4). However, 58 of the up-regulated genes had a $\log_2 FC \geq 3.0$, and 4 of the

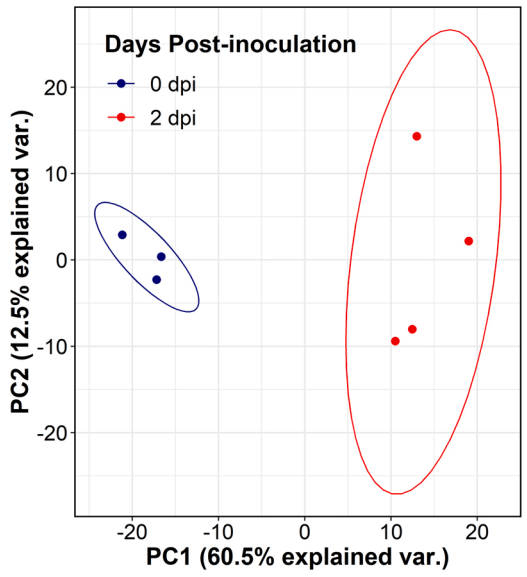

Fig. 3. Turkey blood leukocyte transcriptome variation explained by *Salmonella enterica* serovar Typhimurium UK1 challenge.
Principal component analysis (PCA) was performed on log-transformed normalized read counts per million (cpm) from the 500 genes with highest between-sample variance. The relative positions of each sample on principal component 1 (PC1) and 2 (PC2) are shown for 0 (blue) and 2 (red) days post-inoculation (dpi). Ellipses represent a 95 % confidence interval.

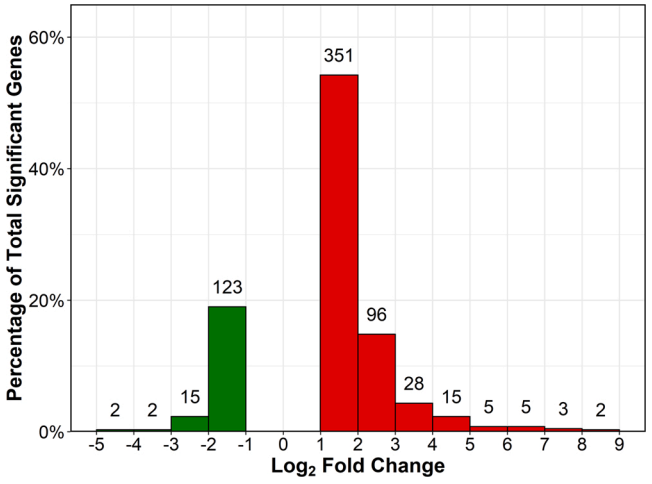

Fig. 4. Distribution of significant gene expression responses to *Salmonella enterica* serovar Typhimurium UK1 in turkey blood leukocytes.
Genes with significant differential expression ($|\log_2FC| \geq 1.0$, FDR < 0.01) at 2 days post-inoculation (dpi) compared to 0 dpi were determined using edgeR methods (Robinson et al., 2010). Each bar shows the percentage of significant genes (out of 647 total) within each range of $\log_2$ fold change; the gene count within that range is listed above the bar. Red = up-regulated genes, green = down-regulated genes.

down-regulated genes had a $\log_2FC \leq$ -3.0 (Fig. 4; **Supplemental Table S2**). A C-C motif chemokine 3-like gene (*LOC100545600*, by homology *CCAH221*) was the most up-regulated gene ($\log_2FC = 8.83$) in leukocytes from the *Salmonella*-challenged turkeys, whereas potassium voltage-gated channel subfamily A member regulatory beta subunit 1 (*KCNAB1*) was the most down-regulated gene ($\log_2FC = -4.53$) (Table 1, **Supplemental Table S2**).

Table 1
Top 10 up-regulated and down-regulated genes in turkey blood leukocytes in response to *Salmonella enterica* serovar Typhimurium UK1.

Gene Symbol	NCBI Gene ID	Gene Description	Chicken Ortholog[a]	$\log_2FC$
LOC100545600	100545600	C-C motif chemokine 3-like	*CCAH221*	8.83
IL4I1	100540143	interleukin 4 induced 1		8.81
LYZ	100547044	lysozyme		7.98
GPM6B	100542981	glycoprotein M6B		7.42
IL13RA2	100545119	interleukin 13 receptor subunit alpha 2		7.27
LOC100540154	100540154	carbonic anhydrase 9-like	*CA9*	6.90
KLF5	100549404	Kruppel like factor 5		6.78
LOC104917577	104917577	vascular endothelial growth factor receptor 1-like	*FLT1*	6.24
LOC100541042	100541042	cytochrome P450 7B1	*CYP7B1*	6.20
IL22RA2	100550792	interleukin 22 receptor subunit alpha 2		6.05
KCNAB1	100544034	potassium voltage-gated channel subfamily A member regulatory beta subunit 1		−4.53
DAAM2	100548077	dishevelled associated activator of morphogenesis 2		−4.38
ADAMTS6	104914924	ADAM metallopeptidase with thrombospondin type 1 motif 6		−3.46
SCG3	100539566	secretogranin III		−3.14
LOC104911574	104911574	uncharacterized loci	—	−2.96
LOC104914168	104914168	serine/arginine repetitive matrix protein 2-like	—	−2.69
LOC100544965	100544965	macrophage mannose receptor 1-like	*MMR1L2*	−2.69
RARB	100545327	retinoic acid receptor beta		−2.56
LOC104911846	104911846	DNA annealing helicase and endonuclease ZRANB3-like	*ZRANB3*	−2.56
LOC100542110 (*CAPRIN2*)	100542110	caprin-2		−2.49

Genes with significant differential expression ($|\log_2FC| \geq 1.0$, FDR < 0.01) at 2 days post-inoculation (dpi) compared to 0 dpi were determined using edgeR methods (Robinson et al., 2010). The top 10 up- and down-regulated genes based on largest $\log_2$ fold change ($\log_2FC$) are shown. See **Supplemental Table S2** for the complete list of all significant genes.
[a] For unannotated genes, orthologous genes in *Gallus gallus* were identified by BLASTP.

3.3. Pathway and functional prediction of blood leukocytes responses to S. Typhimurium UK1

Pathway analysis on the significant DE genes was performed using Ingenuity Pathway Analysis (IPA) software. Using gene symbols obtained from turkey plus orthologs in chicken and human, 601 of the significant DE genes (92.9 %) were recognized by IPA and were significantly associated (FDR < 0.05) to 132 canonical pathways. The pathway associations with highest significance (largest $-\log_{10}$FDR) were the "STAT3 Pathway", "IL-12 Signaling and Production in Macrophages", and "Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses" pathways (Fig. 5**A; Supplemental Table S3**). To predict the downstream effects of the DE genes in the top 3 pathways, functions were associated ($P < 0.05$) with the significant DE genes in each pathway (**Supplemental Fig. S1**). To summarize these effects, the

A.

Top Canonical Pathways (by -log10FDR)	-log10FDR	z-score
STAT3 Pathway	7.08	1.73
IL-12 Signaling and Production in Macrophages	5.88	N/A
Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	4.99	3.05
Neuroinflammation Signaling Pathway	4.75	2.86
Interferon Signaling	4.75	2.12
IL-6 Signaling	4.35	2.14
Prolactin Signaling	4.27	1.27
Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	4.16	2.50
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	4.16	N/A
Th1 and Th2 Activation Pathway	4.16	N/A

-log10FDR 0.0 7.1

Activation z-score 0.0 3.1 N/A

B.

Top Downstream Functions (by z-score)	-log10FDR	z-score
Migration of cells	13.95	5.50
Chemotaxis	9.67	5.09
Cell movement of tumor cell lines	7.75	4.99
Homing of cells	11.51	4.93
Activation of cells	11.13	4.91
Cell movement	15.79	4.86
Migration of tumor cell lines	5.24	4.79
Cell survival	7.68	4.46
Cell viability	6.27	4.42
Cellular homeostasis	16.67	4.40

-log10FDR 0.0 16.7

Activation z-score 0.0 5.5

Fig. 5. Top 10 Ingenuity Pathway Analysis (IPA) associations for turkey blood leukocyte responses to *Salmonella enterica* serovar Typhimurium UK1. Significant associations (FDR < 0.05) were determined in IPA. **A.** Top 10 canonical pathways by largest -log$_{10}$FDR. **B.** Top 10 downstream functions by largest |z-score|. Purple = -log$_{10}$FDR, orange = predicted activation (z-score ≥ 2.0), white = no direction predicted (|z-score| < 2.0), gray = prediction not available (N/A). See **Supplemental Table S3** for all significant IPA associations.

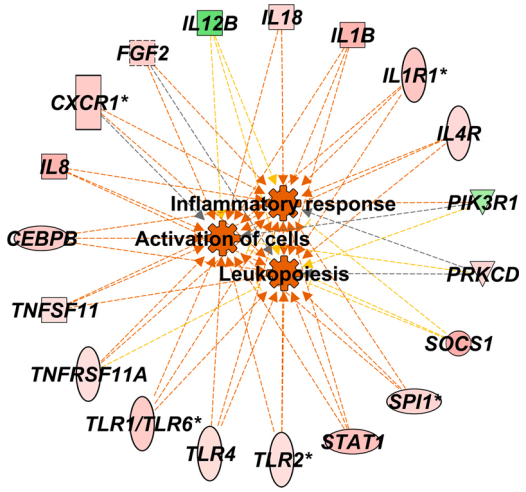

Fig. 6. Differential expression in top canonical pathways predicted to activate immune functions in response to *Salmonella enterica* serovar Typhimurium UK1. The subset of significant differentially expressed (DE) genes in the top 3 IPA canonical pathways (most significant by -log$_{10}$FDR; see Fig. 5, **Supplemental Table S3**) were associated to downstream functions ($P < 0.05$) to identify their predicted effects. Shown are the top 3 downstream functions (most significant by *P*-value) for the subset and the genes linked to all 3 functions. Color illustrates the magnitude of DE for each gene (red = up-regulated genes, green = down-regulated genes) and the predicted relationships with each function (orange = predicted activation, yellow = direction of DE does not support the downstream prediction, gray = no prediction). Official symbols for turkey genes marked with * are *CXCR1* = *LOC100540439*, *IL1R1* = *LOC100542828*, *SPI1* = *LOC104911109* and *LOC100548288*, *TLR2* = *LOC104910743*, *TLR1/TLR6* = *LOC100551461* and *LOC104910799*.

45 genes from the aforementioned three pathways were combined and the top downstream functions by significance (smallest *P*-values) were "leukopoiesis", "activation of cells", and "inflammatory response" (Fig. 6). Among the genes linked to all three of these functions were multiple toll-like receptors (TLRs; toll-like receptor 4 (*TLR4*), toll-like receptor 2 type 1 (*TLR2A*, official symbol *LOC104910743*), toll-like receptor 1 (*LOC100551461*, by homology *TLR1B/TLR6*), and toll-like receptor 6 (*LOC104910799*, by homology *TLR1A/TLR6*)), and cytokines (interleukin 1 beta (*IL1B*), interleukin 8 (*IL8*), interleukin 12 beta (*IL12B*), interleukin 18 (*IL18*) and tumor necrosis factor superfamily member 11 (*TNFSF11*)).

Collectively, the 601 significant DE genes recognized by IPA were also associated (FDR < 0.05) to more than 500 downstream functions. Therefore, the downstream functions were further filtered for directionality using z-score, which gave 180 functions predicted to be activated (z-score ≥ 2.0) and 5 predicted to be inhibited (z-score ≤ 2.0) (**Supplemental Table S3**). As befitting for circulating leukocytes responding to an invasive bacterial pathogen, the strongest predictions (largest |z-score|) were activation of functions related to cell movement, including "migration of cells" ($n = 161$ genes) and "chemotaxis" ($n = 57$ genes) (Fig. 5B, **Supplemental Table S3**). Over 100 of the activated downstream functions were specific to immune cells and included not only cell movement, but also cell activation, proliferation, death, and maintenance terms (**Supplemental Fig. S2, Supplemental Table S3**).

The DE in response to *S*. Typhimurium was also used to predict 17 genes that play an upstream regulatory role (FDR < 0.05, |z-score| ≥ 2.0) and collectively could impact expression of 149 of the significant DE genes (including the 17 predicted regulators). The top predicted upstream regulators (largest |z-score|) from within the significant DE genes included *IL1B*, signal transducer and activator of transcription 1 (*STAT1*) and interferon regulatory factor 7 (*IRF7*), all of which encode proteins strongly predicted (z-score > 4.0) to activate target genes linked to immune-related functions (**Supplemental Table S3**). The regulatory network for *IL1B* included 76 target DE genes, for which the top immune functions by significance were "quantity of leukocytes", "leukopoiesis", and "inflammatory response" (Fig. 7). A vascular endothelial growth factor receptor 1-like gene (*LOC104917577*, by homology *FLT1*), and C-X-C motif chemokine ligand 12 (*CXCL12*) were the most up-regulated genes that contributed to the predicted activation of all three of the top functions, while *IL12B* was the only IL1β-regulated gene with decreased expression that was linked to all three functions.

4. Discussion

Colonization of poultry by non-typhoidal *Salmonella* serovars such as *S*. Typhimurium is associated with human foodborne disease. This study identified the colonization patterns and transcriptional responses of 3-week-old turkeys challenged with the *S*. Typhimurium UK1 strain. Our results demonstrate that this strain was able to effectively colonize the gastrointestinal tract, yet did not produce clinical signs of infection in the birds. This study also utilized RNA-seq to investigate transcriptional changes in turkey peripheral blood leukocytes ($n = 3$ and $n = 4$, for 0 dpi and 2 dpi, respectively). Past investigations have demonstrated that similar sample sizes (n = 3–4 per treatment group) were sufficient to identify DE genes in both commercial chickens (Cui et al., 2017; Park et al., 2017; Sah et al., 2018) and commercial turkeys (Monson et al., 2015; Reed et al., 2018). In the current study, this sample size was also adequate to identify DE genes in turkey leukocytes after *S*. Typhimurium challenge. In fact, the relatively large number (647) of significant DE genes at 2 dpi compared to 0 dpi and the large separation of the pre- and post-inoculation samples by PCA suggest that the differences between these groups are statistically and biologically significant. As turkey lymphocytes and heterophils are functionally mature by 2–3 weeks of age (Lowry et al., 1997; Suresh et al., 1993), the observed DE should predominantly reflect the response to challenge rather than the two

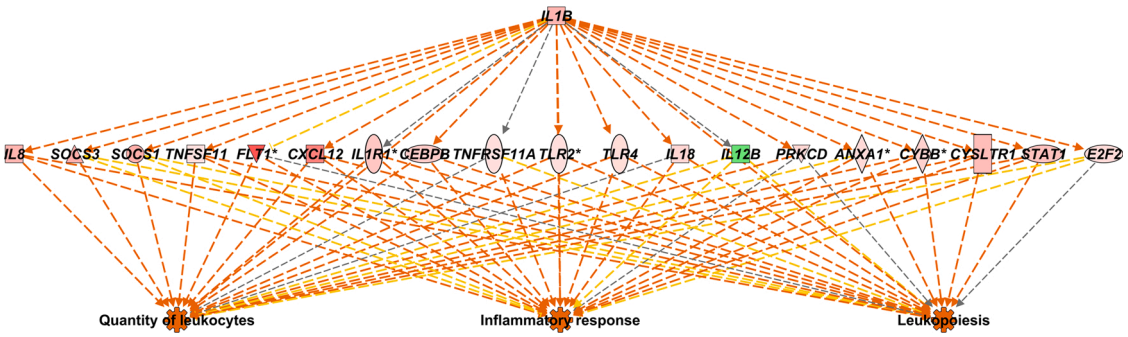

Fig. 7. Predicted activation in IL1β regulatory network in response to *Salmonella enterica* serovar Typhimurium UK1.
IPA identified interleukin 1 beta (*IL1B*) as the most significant upstream regulator (by largest |z-score|) within the significant DE genes (see **Supplemental Table S3** for the full list of predicted upstream regulators). The significant DE genes regulated by IL1β were used to generate associations with downstream functions ($P < 0.05$). The target DE genes associated with all 3 of the top immune-related functions (most significant by *P*-value) are shown. Color illustrates the magnitude of DE for each gene (red = up-regulated genes, green = down-regulated genes) and the predicted relationships between genes/functions (orange = predicted activation, yellow = direction of DE does not support the downstream prediction, gray = no prediction). Official symbols for turkey genes marked with * are *ANXA1* = *LOC100542970*, *CYBB* = *LOC100543754*, *FLT1* = *LOC104917577*, *IL1R1* = *LOC100542828*, *TLR2* = *LOC104910743*.

additional days of age.

Transcriptome and pathway analysis support the hypothesis that following acute challenge with *S*. Typhimurium UK1 in turkeys, circulating leukocytes were activated and had the potential to migrate to tissues and respond to the microbes. Tissues of the intestinal tract and spleen were colonized with *Salmonella* at 7 dpi, but it is unknown whether *S*. Typhimurium UK1 had disseminated systemically at 2 dpi when the whole blood samples were taken for transcriptome analysis. Other strains of *S*. Typhimurium have been detected in the spleen of chickens as early as 1–3 dpi (Lacharme-Lora et al., 2019; Sivula et al., 2008). The predicted pathways and functions for turkey leukocytes suggest that their transcriptional responses were specific to the challenge. These conclusions were based on sequencing of a mixed population of peripheral blood leukocytes, so additional animal experiments and assays (*e.g.*, cell sorted or single-cell RNA-seq) would be needed to identify which immune cell types are responsible for the DE genes following *S*. Typhimurium UK1 inoculation. Future single-cell RNA-seq could also help distinguish the DE due to leukocyte activation (changes in cell state) *versus* leukocyte migration (changes in cell population structure). Cell activation, migration, phagocytosis, and proliferation in response to *Salmonella*, as predicted by our transcriptomic analysis, have been observed previously in different turkey leukocyte populations (Genovese et al., 1998; He et al., 2008; Hesse et al., 2016; Potter et al., 2016). For example, *in vitro* exposure to *Salmonella enterica* serovar Enteritidis (*S*. Enteritidis) increased nitric oxide production in turkey monocytes and oxidative burst and degranulation of heterophils (He et al., 2008), while *in vivo* T lymphocytes migrated into the cecal mucosa of turkeys following colonization by *S*. Typhimurium or *S*. Enteritidis (Hesse et al., 2016).

The largest transcriptional response to *S*. Typhimurium UK1 in this study was increased expression of a C-C motif chemokine 3-like gene (*LOC100545600*), which could be predicted by homology to be the ortholog to chicken chemokine ah221 (*CCAH221*). *CCAH221* has been shown to increase gene expression in response to *S*. Enteritidis in chicken spleen and cecum (Matulova et al., 2013, 2012; Zhou and Lamont, 2007). Although over 80 % of the unannotated turkey genes (with *LOC* number genes symbols) with significant DE were identified using chicken, six of the significant DE genes lacked a chicken ortholog. This illustrates the comparative immunological differences that exist between turkeys and chickens, and that host response to *Salmonella* warrants examination in multiple poultry species.

The STAT3 pathway was strongly associated by IPA with the DE in turkey leukocytes 2 dpi with *S*. Typhimurium UK1, although *STAT3* itself was just below significance ($\log_2$FC = 0.93). In mammals, STAT3 signaling has been shown to stimulate proliferation of granulocytes, to regulate development of lymphocytes, and contrastingly, to have anti-inflammatory functions such as suppression of TLR-induced signal transduction (Hillmer et al., 2016). Different strains of *S*. Typhimurium can trigger STAT3 to inhibit immune responses and facilitate infection (Jaslow et al., 2018; Ruan et al., 2017). Our data do not indicate whether STAT3 signaling had pro- or anti-inflammatory effects in the turkey leukocytes. However, some of the largest DE in this canonical pathway included secreted decoy receptors, interleukin 13 receptor subunit alpha 2 (*IL13RA2*) and interleukin 22 receptor subunit alpha 2 (*IL22RA2*), which can reduce responses to IL13/IL4 and IL22, respectively (Rahaman et al., 2002; Xu et al., 2001). These data suggest that circulating leukocytes were activated in response to *Salmonella*, but also that an increased expression of immunoregulators may be limiting immunopathology and potentially contributing to the lack of noticeable clinical signs.

Turkey leukocyte responses to *S*. Typhimurium UK1 were predicted to include IL12 signaling and production in macrophages. The heterodimeric cytokine IL12, composed of p35 and p40 subunits, is an important activator of T cell proliferation and Th1 responses to intracellular pathogens; for example, IL12 has been shown to induce interferon gamma production and splenocyte proliferation in chickens (Degen et al., 2004). *IL12B*, which encodes the p40 subunit of both IL12 and IL23 (Truong et al., 2017), is up-regulated at various dpi in chicken tissues in response to *S*. Typhimurium (Bai et al., 2014; Berndt et al., 2007; Dar et al., 2019; Wang et al., 2019). The majority of genes associated with the IL12 cytokine signaling pathway were up-regulated in turkey leukocytes at 2 dpi and were predicted to activate cell proliferation, although expression of *IL12B* itself decreased. This observation may reflect that 2 dpi missed (either early or late) any positive response in *IL12B*, or that circulating cells do not respond the same as tissue-resident immune cells. Further investigation of IL12 expression across multiple timepoints and tissues in turkeys would clarify the dynamics of its response to *Salmonella*.

Predicted up-regulation of pattern recognition receptor (PRR) signaling in turkey leukocytes exposed to *S*. Typhimurium UK1 would have immune-activating effects, although these effects remained subclinical. Activation of PRR signaling included increased expression of the toll-like receptors *TLR4*, which recognizes bacterial lipopolysaccharide (LPS), and *TLR1A/TLR1B* and *TLR2A*, which form heterodimers recognizing tri- or di-acetylated lipoproteins from bacteria (Keestra et al., 2013; Neerukonda and Katneni, 2020). These TLRs activate NF-κB through MyD88 signaling and initiate pro-inflammatory responses (Keestra et al., 2013; Neerukonda and Katneni, 2020). Also up-regulated in turkey leukocytes after exposure to *S*. Typhimurium UK1 was toll-like receptor 15 (*TLR15*, official symbol *LOC100542515*), which recognizes

microbial proteases, but is not present in the IPA knowledgebase because it is unique to birds and reptiles (Keestra et al., 2013; Neerukonda and Katneni, 2020). Inoculation with *S*. Typhimurium also impacted expression of *TLR1A*, *TLR4*, and *TLR15* in the chicken intestine (Higgs et al., 2006; Shaughnessy et al., 2009; Wang et al., 2019). Related to PRR responses, *IL1B* was up-regulated by *S*. Typhimurium UK1 and predicted by IPA to be an important regulator of other genes with immune-activating DE, including the TLRs (*TLR4* and *TLR2*) and other cytokines (*IL8* and *IL18*), and to be associated with inflammation and leukocyte proliferation. Likely expressed by monocytes and macrophages, up-regulation of *IL1B* after *S*. Typhimurium challenge has been demonstrated across chicken tissues and cells (Bai et al., 2014; Beal et al., 2004; Fasina et al., 2008; Huang et al., 2020; Shaughnessy et al., 2009; Withanage et al., 2004; Zhang et al., 2020) and has pro-inflammatory effects on lymphocytes and heterophils to activate them against *Salmonella*.

Contrary to the robust DE of genes in turkey leukocytes in response to *S*. Typhimurium UK1, challenge of 3-week old turkeys with either a foodborne outbreak strain of *Salmonella enterica* serovar Heidelberg (*S*. Heidelberg) or an attenuated vaccine strain of *S*. Typhimurium induced only minimal DE in peripheral leukocytes at 2 dpi (Bearson et al., 2017, 2019). Responses to both the *S*. Typhimurium vaccine and *S*. Heidelberg lacked the immune-activation characteristic of the transcriptomic response to *S*. Typhimurium UK1. Likely contributing to the differences in response to these strains, higher tissue colonization levels (1–2 logs) and greater fecal shedding (2–3 logs) were observed in the *S*. Typhimurium UK1 challenged turkeys compared to the turkeys challenged with *S*. Heidelberg or immunized with the vaccine.

Despite the differences between these turkey challenge studies, over half of the genes with changes in expression after inoculation with *S*. Heidelberg (10 of 18) or the *S*. Typhimurium vaccine (19 of 26) were also differentially expressed after challenge with *S*. Typhimurium UK1 (Bearson et al., 2017, 2019). Two of the most down-regulated genes were conserved across all three strains (secretogranin III (*SCG3*) and *KCNAB1*). SCG3 participates in regulation and biogenesis of secretory granules in endocrine cells (Gomi et al., 2015). *KCNAB1* encodes a voltage-gated potassium channel that based on its gene family members could play a role in naïve leukocyte activation and differentiation to memory cells (Chandy et al., 2004). The only inconsistent DE observed was for the aconitate decarboxylase 1 (*ACOD1*) gene, which was down-regulated by the *S*. Typhimurium vaccine (Bearson et al., 2019), but highly up-regulated by pathogenic *S*. Typhimurium UK1. Two studies in chickens demonstrated that *ACOD1* was strongly up-regulated in spleen and cecum after challenge with *S*. Enteritidis (Matulova et al., 2013, 2012). Mammalian ACOD1 indirectly decreases TLR-induced expression of pro-inflammatory cytokines (Li et al., 2013), but also has antimicrobial activity by producing itaconic acid (Michelucci et al., 2013). Neither *S*. Typhimurium nor *S*. Heidelberg resulted in clinical signs in the birds (no change in cloacal temperatures; no morbidity or mortality), yet these different *Salmonella* serovars induced distinct host responses in turkeys. Overall, these data illustrate the challenge of understanding sub-clinical colonization, and provide genes and pathways that could be targeted in future investigations.

4.1. Conclusions

In summary, inoculation of 3-week-old turkeys with *S*. Typhimurium UK1 resulted in asymptomatic colonization of the gastrointestinal tract and systemic dissemination to the spleen. Contrary to the lack of detectable clinical signs of infection in the turkeys following challenge, this human foodborne pathogen elicited an immune-activating transcriptional response in circulating leukocytes, which included the STAT3 pathway, as well as cytokine and toll-like receptor signaling. Among the 647 genes with significant DE in response to *S*. Typhimurium UK1 challenge, IL1β was predicted as a major activator of inflammatory gene expression, while the strong increases in *CCAH221*, *IL13RA2*, *IL22RA2*, and *ACOD1* could also act to modulate the immune response. As these DE genes were obtained from a single acute time point, future research needs to determine whether the transcriptional responses in turkeys to *S.* Typhimurium persist at later time points and could therefore have potential as diagnostic markers. Understanding host responses in blood leukocytes to *S*. Typhimurium may benefit the design of new or improved mitigation strategies (*e.g.*, vaccines, immunomodulators, *etc.*) that optimize the turkey immune response to control colonization, dissemination, and/or persistence of *Salmonella*, with the long-term goal of assisting producers in reducing the presence of *Salmonella* in commercial poultry flocks for improved food safety.

Author contributions

Authors SB and BB designed the experiments and acquired the experimental data. SB, MM, MS, BB and TL performed data analysis and interpretation. The manuscript was drafted and revised for important intellectual content by SB, MM, MS, BB, SL and TL, as well as, final approval of the version to be published with agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Data availability

RNA-seq datasets generated and analyzed for this study are available in the NCBI GEO database under accession number GEO: GSE151896. Results of the DE and IPA analyses are provided in the supplemental files and shedding/colonization data are available from the corresponding author upon reasonable request.

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Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetimm.2020.110181.

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